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TITLE: OZONE INDUCED FORMATION OF O O' **DI TYROSINE** CROSS LINKS IN PROTEINS.
AUTHOR(S): VERWEIJ H; CHRISTIANSE K; VAN STEVENINCK J
CORPORATE SOURCE: SYLVIUS LAB., DEP. MED. BIOCHEMISTRY, WASSENAARSEWEG 72, 2333 AL LEIDEN.
SOURCE: **BIOCHIM BIOPHYS ACTA, (1982) 701 (2), 180-184.**

2. TITLE: CHEMICAL NATURE OF MONOGENEAN SCLERITES PART 1 STABILIZATION OF CLAMP **PROTEIN** BY FORMATION OF **DI TYROSINE.**
AUTHOR(S): RAMALINGAM K
SOURCE: PARASITOLOGY, (1973) 66 (1), 1-7.
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TITLE: CD and proton NMR studies on the side-chain conformation of tyrosine derivatives and tyrosine residues in di- and tripeptides
AUTHOR(S): Juy, Michel; Lam Thanh Hung; Fermandjian, Serge
CORPORATE SOURCE: Dep. Biol., Cent. Nucl. Stud., Gif-sur-Yvette, 91191, Fr.
SOURCE: **International Journal of Peptide & Protein Research (1982), 20(4), 298-307**

3. Journal of the American Chemical Society (1985), 107(3), 659-66

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5. Salt-**stabilized protein** formulation

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Thank you !

Tekchand Saidha
Primary Examiner
Art Unit 1652, CM1, Room No. 10D05
Mail Box 10D01
(703) 305-6595

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OZONE-INDUCED FORMATION OF *O,O'*-DITYROSINE CROSS-LINKS IN PROTEINS

HENDRIK VERWEIJ, KARMINAH CHRISTIANSE and JOHNNY VAN STEVENINCK

Sylvius Laboratories, Department of Medical Biochemistry, Wassenaarseweg 72, 2333 AL Leiden (The Netherlands)

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Key words: Cross-linking; Ozone; Dityrosine

Treatment of spectrin, insulin, glucagon and ribonuclease with ozone results in covalent cross-linking of these proteins. This cross-linking is not reversed by treatment with dithiothreitol and thus can not be ascribed to -S-S- bond formation. A concomitant *O,O'*-dityrosine formation is observed by spectrofluorometric analysis of the protein and by amino acid analysis and thin-layer chromatography of hydrolyzed protein samples. It is highly probable that the observed protein cross-linking should be attributed to interpeptide *O,O'*-dityrosine bonds. Several authors have shown before that oxidation of proteins with horseradish peroxidase and H_2O_2 also leads to *O,O'*-dityrosine formation. Peroxidase-induced *O,O'*-dityrosine formation in galactose oxidase (D-galactose:oxygen 6-oxidoreductase, EC 1.1.3.9) causes a strong increase of enzyme activity. In accordance with these observations ozone treatment of galactose oxidase also leads to *O,O'*-dityrosine formation with a concomitant 8-fold increase of enzyme activity.

Introduction

Various toxic effects of ozone on biological systems have been described in recent literature, including deleterious effects on lung tissue [1–6], membrane damage of erythrocytes [7–10] and inhibition of several enzymes [11–14]. In attempts to explain ozone toxicity on a molecular level it has been shown that ozone may cause, among other things, lipid peroxidation [12,13,15] and covalent protein cross-linking [12,16]. It has been suggested that this ozone-induced cross-linking is caused by a reaction of proteins with lipid peroxidation aldehyde products [12], but in recent studies we have shown that this hypothesis is untenable. One of the main arguments was that ozone treatment of solubilized, lipid-free spectrin caused extensive cross-linking [16]. Further it was shown that the mechanisms of ozone-induced and photodynamic protein cross-linking are quite different [17,18].

In further experiments it was tried to elucidate the molecular background of ozone-induced protein cross-linking. As shown in the present paper, ozone treatment of proteins leads to *O,O'*-dityrosine generation. Therefore it seems highly probable that ozone-induced protein cross-linking should be ascribed to the formation of interpeptide dityrosine bridges.

Materials and Methods

Galactose oxidase (D-galactose:oxygen 6-oxidoreductase, EC 1.1.3.9) and insulin were obtained from Sigma, ribonuclease from Boehringer and glucagon from Serva. All other reagents were analytical grade and used without further purification. Ozone was generated with a Supelco Micro-Ozonizer in oxygen, at a rate of 2.5 μmol · min^{-1}. Protein and tyrosine solutions were exposed to 0.1–2.5 μmol O_3 per min by bubbling the gas into 3-ml samples.

Spectrin was isolated from human red blood cells as described before [17]. Protein concentrations were measured by the method of Lowry et al.

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[19], with bovine serum albumin as a standard. Preparation of *O,O'*-dityrosine was performed according to the method of Malanik and Ledvina [20].

Gel electrophoresis of spectrin and ribonuclease was done on sodium dodecyl sulphate (0.2%)-polyacrylamide (5.6% w/v) gels as described by Fairbanks et al. [21]. Gel electrophoresis of insulin and glucagon was done on 7.5% acrylamide gels (with an acrylamide/bisacrylamide ratio of 15:1) in the presence of 8 M urea, as described by Swank and Munkres [22]. Densitometric scans of Coomassie blue stained gels were recorded on a Zeiss PMQII spectrophotometer with a scanning device. Protein cross-linking was estimated by the scan patterns.

Amino acid analysis was done on 22-h 6 M HCl hydrolysates, using a Beckman amino acid analyzer, equipped with a Dowex 50 column (40 × 0.4 cm). The gradient elution procedure as described by Malanik and Ledvina [23] was followed. Before hydrolysis galactose oxidase was converted to the apo-enzyme as described by Tressel and Kosman [24]. Thin-layer chromatography of hydrolysates was done on silica gel plates, according to the method of Aeschbach et al. [25], utilizing the solvent systems butanol-1/acetic acid/water (4:1:1, v/v) and propanol-1/ammonia 25% (7:3, v/v).

Fluorescence measurements were carried out on an Aminco Bowman spectrofluorometer. Galactose xidase was assayed by measuring oxygen consumption as described by Tressel and Kosman [24].

Results

When spectrin was exposed to ozone, a progressive polymerization of the protein occurred, as shown in Fig. 1. Two polymerized bands were visible: one with a molecular weight between 400 and 500 kdaltons (apparently a spectrin dimer) and a second product with a molecular weight higher than 500 kdaltons, on top of the gel. This cross-linking could not be attributed to -S-S- bond formation, as the protein samples were treated with dithiothreitol prior to electrophoresis. A similar cross-linking was observed with insulin, glucagon and ribonuclease, but n t with galactose oxidase (n t shown).

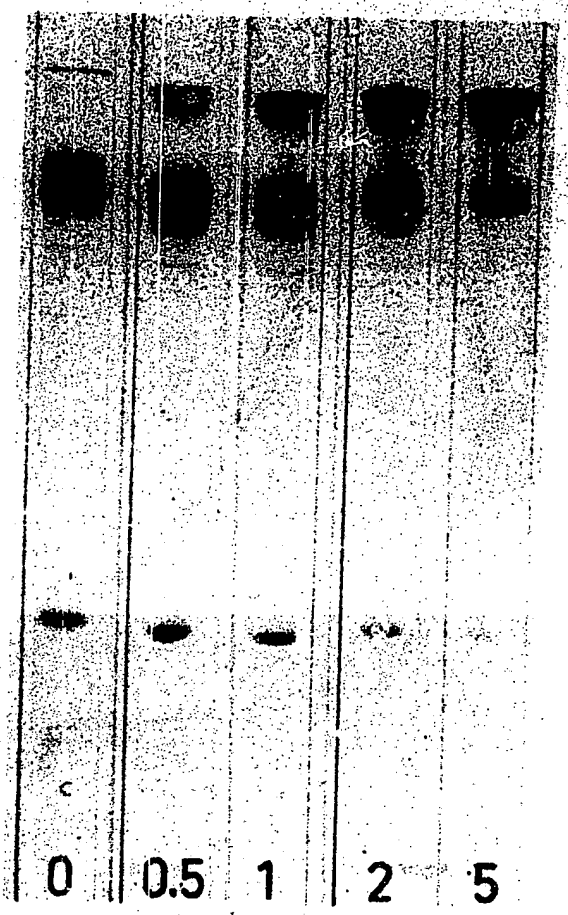

Fig. 1. Ozone-induced cross-linking of spectrin as revealed by gel electrophoresis. 3 ml spectrin solution (1.0 mg/ml) in 10 mM phosphate buffer, pH 7.0, were treated with ozone (2.5 μmol/min), as described in the Materials and Methods. Exposure times in min as indicated.

Simultaneously the protein exhibited increasing fluorescence with an emission maximum of about 415 nm, when excited with light of 330 nm (Fig. 2). The fluorescence was not influenced by acid hydrolysis of the protein. A similar fluorescence was observed by Aeschbach et al. in proteins exposed to horseradish peroxidase and H_2O_2 [25]. These authors have shown that this fluorescence was caused by peroxidase-induced formation of *O,O'*-dityrosine in these proteins. In accordance, exposure of spectrin to peroxidase/H_2O_2, as described by Aeschbach et al., yielded a fluorescence indistinguishable from the ozone-induced fluorescence (Fig. 2). These results strongly suggest the presence of *O,O'*-dityrosine in spectrin after exposure to O_3.

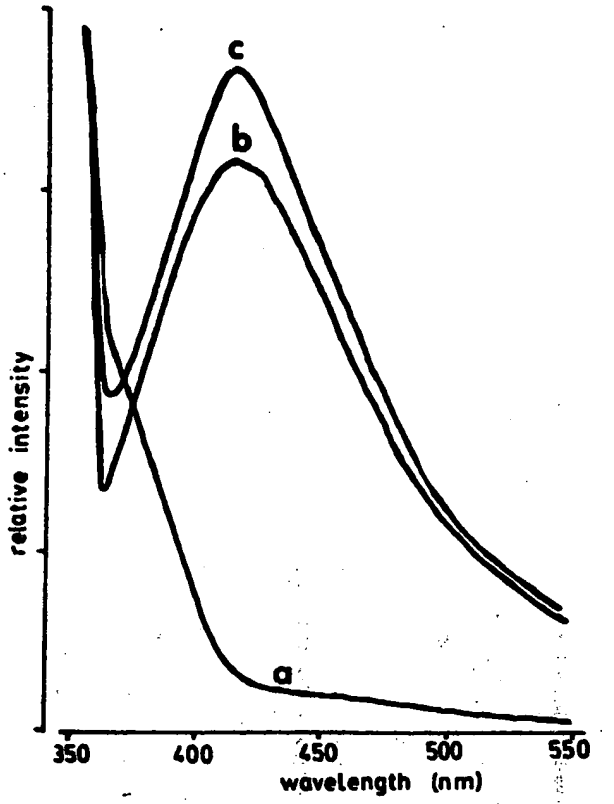

Fig. 2. Fluorescence emission spectra of (a) untreated spectrin, (b) spectrin, treated during 5 min with ozone and (c) spectrin, 1 mg/ml 0.1 M borate buffer, pH 10, incubated during 5 min with 25 mM H_2O_2 in the presence of 100 U horseradish peroxidase/ml.

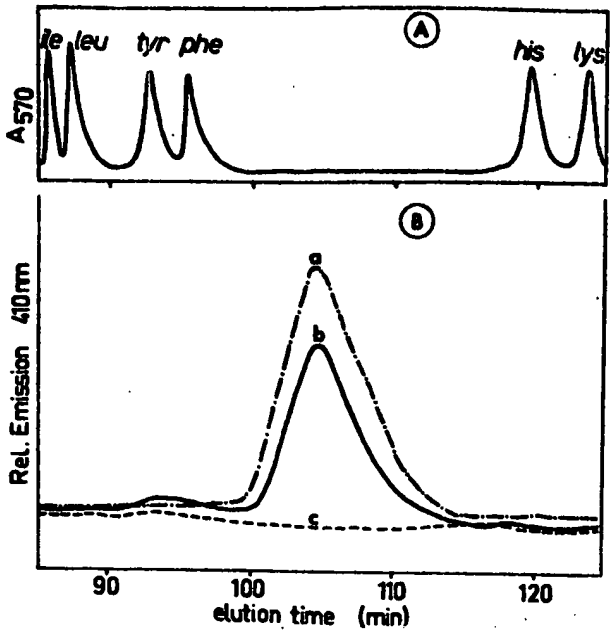

Fig. 3. Section of amino acid chromatograms of (A) standard amino acid mixture, detection by ninhydrin absorbance and (B) detection by fluorescence at pH 10.0 in the eluted fractions of hydrolyzed samples of (a) *O,O'*-dityrosine, (b) ozone-treated spectrin and (c) untreated spectrin.

The presence of *O,O'*-dityrosine in ozone-treated protein was confirmed by thin-layer chromatography. With both solvent systems described in Materials and Methods a fluorescent spot, with the same R_F value as purified *O,O'*-dityrosine, was found in the hydrolysates of the oxidized proteins.

Fig. 3 shows amino acid chromatograms of hydrolyzed spectrin and of a standard mixture. Again *O,O'*-dityrosine could be identified after exposure of the protein to ozone. Also with ribonuclease, glucagon and insulin, *O,O'*-dityrosine could only be detected after ozone exposure. With galactose oxidase the results were slightly different. Even the untreated protein contained a very small amount of *O,O'*-dityrosine, that increased significantly during exposure f the protein t ozone.

Both ozone-induced cross-linking and *O,O'*-

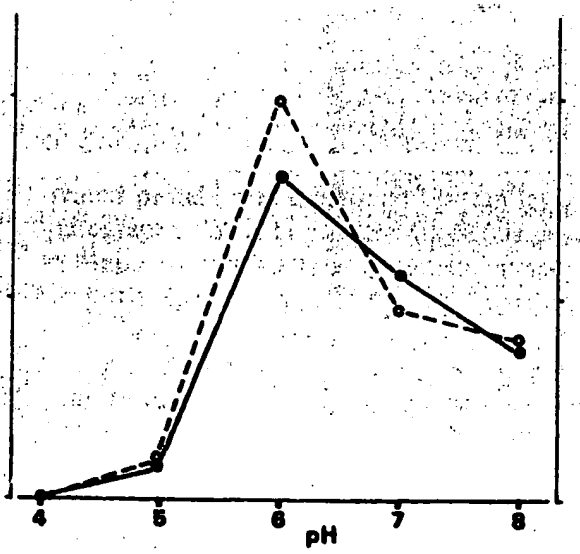

Fig. 4. Ozone-induced *O,O'*-dityrosine formation and cross-linking in spectrin at different pH. Conditions as in Fig. 1. Initial velocities were calculated from the emission intensities and from the amounts of cross-linked protein on top of the gels, as measured by densitometric scanning. ——●, relative velocity f *O,O'*-dityrosine formation: O------O, relative velocity f cross-linking.

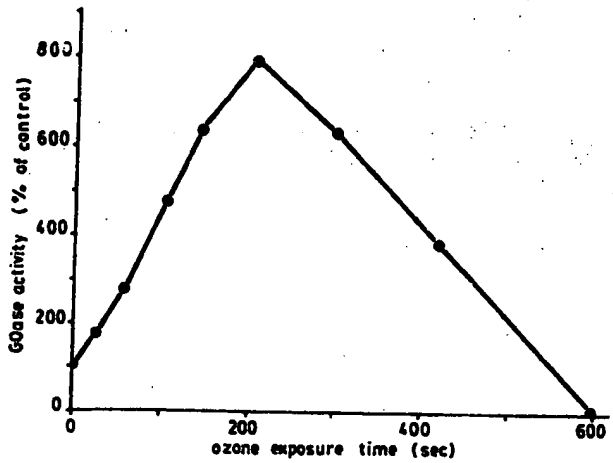

Fig. 5. Effect of ozone on galactose oxidase (GOase) activity, measured as described by Tressel and Kosman [24]. 2.5 ml enzyme solution (0.5 mg/ml) was treated in 10 mM phosphate buffer, pH 7.0, with 0.11 μmol ozone/min.

dityrosine formation are pH-dependent. As shown in Fig. 4, there is a close parallel between the two processes. At pH 4 no cross-linking and no *O,O'*-dityrosine formation were observed. At increasing pH both phenomena varied in a parallel fashion.

The influence of ozone on galactose oxidase activity is depicted in Fig. 5. With small quantities of ozone, enzyme activity was enhanced up to 800%. After longer exposure times the enzyme was inhibited.

Discussion

Cross-linking of red cell membrane proteins by ozone was interpreted by Chan et al. [12] as a reaction of these proteins with aldehyde lipid peroxidation products. In a subsequent study it was demonstrated, however, that cross-linking of spectrin also occurred in the absence of lipids, indicating that cross-linking is caused by a direct effect of ozone on the protein molecule [16].

As shown in Results, exposure of various proteins to ozone leads to *O,O'*-dityrosine formation. The presence of *O,O'*-dityrosine is ozone-treated proteins was conclusively demonstrated by the following observations.

1. The fluorescence spectra obtained with oxidized proteins (Fig. 2) were characteristic for *O,O'*-dityrosine. The excitation maximum of 330 nm and the emission maximum of 410 nm were identical to those of purified *O,O'*-dityrosine and in accordance with previous observations by Anderson [26].

2. Thin-layer chromatography of hydrolysates of oxidized proteins yielded a fluorescent spot with the same R_F value as purified *O,O'*-dityrosine.

3. Amino acid analysis on a Beckman analyzer also showed the presence of *O,O'*-dityrosine in oxidized proteins (Fig. 3).

O,O'-Dityrosine occurs normaly in several structural proteins [26–28]. Presumably the insolubility and elasticity of some of these proteins is, at least partially, caused by these *O,O'*-dityrosine cross-links. Aeschbach et al. described oxidative *O,O'*-dityrosine formation and concurrent cross-linking by exposure of proteins to horseradish peroxidase in the presence of H_2O_2 [25], via a reaction mechanism first described by Gross and Sizer [29]. Apparently ozone causes such a cross-linking by *O,O'*-dityrosine bridges in spectrin, insulin, glucagon and ribonuclease. Quantitative analysis of fluorescence of ozone-treated protein samples, utilizing *O,O'*-dityrosine as a standard, revealed that only a small percentage of the tyrosine residues in ozone-treated proteins was converted to *O,O'*-dityrosine. For instance, in the experiment shown in Fig. 1, the *O,O'*-dityrosine concentration was about 0.5 nmol/nmol spectrin after an exposure time of 2 min. Considering the fact that spectrin contains 44 tyrosine residues per monomer [30] and that each *O,O'*-dityrosine has originated from two tyrosine residues it thus appears that only 2–3% of the tyrosine residues were oxidized at this point of time. Quantitatively this *O,O'*-dityrosine concentration is sufficient to explain the observed cross-linking. Moreover, a strict causal relationship between ozone-induced *O,O'*-dityrosine formation and cross-linking is strongly suggested by the similar pH-dependences of the two phenomena (Fig. 4).

However, other possible cross-linking mechanisms are not rigidly ruled out by the described experimental results. In this context it should be emphasized that several other amino acid residues in proteins (among others, histidine and tryptophan) can be oxidized by ozone [31].

Aeschbach et al. [25] pointed out that peroxidase/H_2O_2-induced *O,O'*-dityrosine formation

leads to both inter- and intramolecular cross-links [25]. It should be expected that this also applies for ozone-induced *O,O'*-dityrosine formation. In this context the observations on galactose oxidase are relevant. In the untreated protein a small amount of *O,O'*-dityrosine was detectable, in agreement with previous observations by Tressel and Kosman [24]. These authors demonstrated that oxidation in the enzyme with peroxidase/H_2O_2 caused a significant increase of *O,O'*-dityrosine content, leading to intramolecular, but not to intermolecular, cross-linking. Further it was shown that this oxidative modification resulted in stimulation of enzyme activity. There is a striking similarity between these observations and the ozone effects on galactose oxidase, as described in this paper. Ozone treatment also resulted in *O,O'*-dityrosine formation, without intermolecular cross-linking, with a concomitant enhancement of enzyme activity (Fig. 5).

To our knowledge enzyme activation by ozone, as demonstrated here for galactose oxidase, has not been described before for other enzymes. Most enzymes are strongly inhibited by ozone, even in very low concentrations [11–14]. It would be premature to attribute this inhibition to inter- or intramolecular *O,O'*-dityrosine cross-link formation, as several other amino acid residues in proteins are also susceptible to ozone-induced oxidation [31].

Acknowledgments

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